Featured Article

Identification of HLA-A24-Restricted CTL Epitope from Cancer-Testis Antigen, NY-ESO-1, and Induction of a Specific Antitumor Immune Response

Hiroshi Yamaguchi, Fumiaki Tanaka, Mitsuhiko Ohta, Hiroshi Inoue, and Masaki Mori

Division of Molecular and Surgical Oncology, Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan

Abstract

Purpose: For the development of peptide-based, cancer-specific immunotherapy, the identification of CTL epitopes from additional tumor antigens is very important. NY-ESO-1, a cancer–testis antigen, is considered to be a promising target of tumor-specific immunotherapy. Because HLA-A24-expressing individuals cover >60% in the population of Japan, we aim at identifying NY-ESO-1-encoded peptide presented by HLA-A24.

Experimental Design: In our study, a HLA-A24-restricted CTL epitope was identified by using the following four-step procedure: (*a*) computer-based epitope prediction from the amino acid sequence of NY-ESO-1 antigen; (*b*) peptide-binding assay to determine the affinity of the predicted peptide with HLA-A24 molecule; (*c*) stimulation of primary T-cell response against the predicted peptides *in vitro*; and (*d*) testing of the induced CTLs toward various carcinoma cells expressing NY-ESO-1 antigen and HLA-A24.

Results: Of the tested peptides, effectors induced by a peptide of NY-ESO-1 at residue position 158–166 lysed three kinds of carcinoma cells expressing both NY-ESO-1 and HLA-A24. Our results indicate that peptide NY-ESO-1 (158–166) (LLMWITQCF) is a new HLA-A24-restricted CTL epitope capable of inducing NY-ESO-1-specific CTLs *in vitro* mediating HLA class I-restricted manner.

Conclusions: We identified a novel HLA-A24-restricted NY-ESO-1-derived epitope peptide (LLMWITQCF) that could induce specific CTLs from the peripheral blood mononuclear cells of HLA-A24[+] healthy donors. This peptide would be useful in further evaluating the clinical utility of peptide-based, cancer-specific immunotherapy against various histological tumors.

Introduction

The cancer–testis (CT) antigen family has been an attractive target for tumor immunotherapy because these antigens can be recognized by human CTLs (1, 2) and are expressed in a variety of malignant tumors of varying histological origin but not in normal tissues, with the exception of the testis and placenta (3). The antigenic peptides derived from CT antigens, such as MAGE-1 (1), MAGE-3 (4), and NY-ESO-1 (5), have been proven to elicit a CTL response in the context of MHC class I molecules. Moreover, immunization with peptide-pulsed dendritic cell (DC) vaccines, as a modality of specific immunotherapy, has been applied to melanoma patients and other malignant patients and found to have some clinical effectiveness (6, 7). However, the expression of CT antigens is heterogeneous among tumors of different histological origins, different patients, and between individual lesions. These characteristics of CT antigen expression suggest that the development of a specific immunotherapy based on as many antigens as possible would be more clinically beneficial than treatment using a single peptide.

The NY-ESO-1 antigen is a classic CT antigen initially identified by serological analysis of recombinant cDNA expression cloning (SEREX) of an esophageal cancer patient (8). NY-ESO-1 mRNA expression is found in 20–30% of melanomas, lung, breast, ovarian, and bladder cancers (8). Recently, Jungbluth *et al.* (9) reported that the expression of NY-ESO-1 is also seen in a high proportion (80%) of synovial sarcomas. On the other hand, HLA-A24 is the most common HLA-A allele among Japanese, with an estimated frequency of 60%, and is also present in Caucasians, with an estimated frequency of 10% (10). Therefore, if NY-ESO-1-derived HLA-A24-restricted CTL epitopes are identified, these peptides may be widely applied for specific immunotherapy against NY-ESO-1-positive tumors in the clinical setting. In this study, we identified a potential peptide derived from the *NY-ESO-1* gene encoding HLA-A24 from the peripheral blood mononuclear cells (PBMCs) of three HLA-A24 healthy donors by *in vitro* stimulation with a NY-ESO-1 synthetic peptide that bound with high affinity to the HLA-A24 molecule and could induce a potent antigen-specific immune response.

Materials and Methods

Cell Lines. The TISI cells, a human B-lymphoblastoid cell line showing HLA-A24, were supplied by Takara Shuzo Co., Ltd. (Otsu, Japan). The gastric carcinoma cell line MKN7 (HLA-A*2402), MKN28 (HLA-A31, HLA-A33), AZ521 (HLA-A*0201, A*0209), and the esophageal carcinoma cell line TE4 (HLA-A*0207, A*1101), TE8 (HLA-A*2601) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University. The colon carcinoma cell line WiDr (HLA-A*2402) and chronic myeloid leukemic cell line K562 were obtained from the Japanese Collection of Research Bioresources Cell Bank. These cell lines were maintained in RPMI 1640 containing fetal bovine serum. The melanoma cell line SK-MEL-128 (HLA-A*2402) was a kind gift from Dr. Lloyd J. Old (Ludwig Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, NY).

Synthetic Peptides. NY-ESO-1 peptides with HLA-A24-binding motifs were predicted by computer analysis as described previously (11). All of the peptides were purchased from Nikka Techno Service Co., Ltd. (Hitachi, Japan). The peptides were isolated and purified by repeated ether precipitations. The purity was determined by analytical reversed phase high-performance liquid chromatography and proved to be $\geq$90% pure. The peptides were dissolved in DMSO and stored at $-30°C$ before use. MAGE-2-derived peptide (EYLQLVFGI) has been shown previously to become a CTL epitope in the context of HLA-A24 (12).

Cytokines. Human recombinant interleukin (IL)-2 was a kind gift from Takeda Pharmaceutical (Osaka, Japan). Human recombinant granulocyte/macrophage colony-stimulating factor, IL-4, and IL-7 were purchased from Genzyme/Techne Corp. (Minneapolis, MN).

Peptide Binding Assay. Peptide binding affinity to HLA-A24 was assessed by a HLA-A24 stabilization assay as described previously (13), based on the findings that MHC class I molecules could be stabilized on the cell surface in the presence of binding peptides. Briefly, RMA-S A*2402/K^b cells are TAP-deficient mouse RMA-S cells (mouse lymphoma cell line) transfected with a chimeric MHC class I cDNA consisting of α1 and α2 domains derived from human HLA-A*2402 molecule, and α3, transmembrane, and intracellular domains derived from mouse H-2K^b molecule (Ref. 14; kindly provided from Dr. H. Takasu, Sumitomo Pharmaceutical, Osaka, Japan). After incubation of the cells in culture medium at 26°C for 18 h, the cells (2×10^5) were washed with PBS and suspended with 1 ml of Opti-MEM (Life Technologies, Inc., Tokyo, Japan) containing 3 μg/ml β2-microglobulin with or without 100 μg of peptide, followed by incubation at 26°C for 3 h and then at 37°C for 3 h. After washing with PBS, the cells were incubated with anti-HLA-A24 monoclonal antibody (mAb; c7709A2.6, kindly provided by Dr. P. G. Coulie, Ludwig Institute for Cancer Research, Brussels Branch) at 4°C for 30 min, followed by incubation with FITC-conjugated rabbit antimouse IgG at 4°C for 30 min. The cells were then suspended with 1 ml of PBS containing 1% formaldehyde and analyzed by FACScan (Becton Dickinson, Mountain View, CA). Binding affinity was evaluated by comparing the mean fluorescence intensity of HLA-A24 expression in the presence of peptide pulsation to the mean fluorescence intensity in the absence of the peptide.

Preparation of Antigen-Presenting Cells and CD8-Positive T Cells from PBMCs. PBMCs were isolated from healthy donors (HLA-A24) by standard density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). PBMCs were incubated in AIM-V medium (Life Technologies, Inc.), supplemented with 2-mercaptoethanol (50 μM) and HEPES (10 mM) for 2 h at 37°C in a culture flask to separate adherent cells and nonadherent cells. Adherent cells were then cultured in the presence of IL-4 (1000 units/ml) and granulocyte macrophage colony-stimulating factor (1000 units/ml) in AIM-V medium for 7 days to generate monocyte-derived DCs. The adherent cells containing DCs were used as antigen-presenting cells [adenomatous polyposis coli (APCs)]. CD8-positive T lymphocytes were isolated from nonadherent cells by the MACS separation system (Miltenyi Biotech, Bergisch Gladbach, Germany) using anti-CD8 mAb coupled with magnetic microbeads according to the manufacturer's instructions. To obtain phytohemagglutinin (PHA)-stimulated blasts, CD8-negative nonadherent PBMCs were cultured in AIM-V medium containing 1 μg/ml PHA (WAKO Chemicals, Osaka, Japan) and 100 units/ml IL-2 for 3 days, followed by washing and cultivation in the presence of IL-2 (100 units/ml) for 4 days.

CTL Induction Using Autologous DCs and PHA-Blasts. CTL induction was performed according to a procedure described previously (15) with a slight modification. Briefly, autologous DCs were treated with mytomycin C (Kyowa Hakko Co., Ltd., Osaka, Japan) and washed with AIM-V medium. DCs were then incubated at room temperature for 2 h in AIM-V with β2-microglobulin (2.5 μg/ml) and peptide (50 μg/ml). On day 1, 1×10^5 peptide-pulsed DCs/well were plated on 24-well plates and cultured with 1×10^6 CD8$^+$ T cells in 2 ml of AIM-V, supplemented with recombinant IL-7 (10 ng/ml). On day 7, 5×10^5 PHA-blasts were treated with mytomycin C, washed twice, pulsed with 50 μg/ml peptides, and then added to each well. On day 8, IL-2 was added to each well at a concentration of 50 units/ml. The peptide stimulation using PHA-blasts as APCs was repeated every 7 days. During CTL induction, cells were fed with fresh AIM-V medium supplemented with IL-2 (50 units/ml) every 3–4 days. On day 28, the cytotoxic activity of T cells was assessed by a conventional 6-h ^{51}Cr release assay.

Reverse Transcription-PCR Analysis of NY-ESO-1 Expression. Total RNA was isolated from the tumor cell lines using the acid guanidinium thiocynate-phenol-chloroform extraction procedure. cDNA was synthesized by reverse transcription from 2.5 μg of total RNA. NY-ESO-1 cDNA was detected by PCR amplification using oligonucleotide primers specific for the different exons of the *NY-ESO-1* gene. The following oligonucleotides were used as primers: sense 5′-CGCCTGCTT-GAGTTCTACCTC-3′ and antisense 5′-AGGGAAAGCTGCT-GGAGACAG-3′. PCR was performed for 30 cycles (1 min at 94°C, 1 min at 59°C, and 1 min at 72°C). The PCR product was size fractionated on a 1% agarose gel. To ensure that the RNA had not degraded, a PCR assay with primers specific for the glyceraldehyde-3-phosphate dehydrogenase cDNA gene was thus carried out in each case.

Establishment of Transient NY-ESO-1-Transfected Cancer Cell Lines. Human NY-ESO-1 cDNA was generated by reverse transcription-PCR and subcloned into pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) as described previously (5) and then transfected into the four cell lines (WiDr, MKN7, TE8, and MKN28) by the Lipofectamine method (Life Technologies, Inc.). The transfectants were incubated at 37°C for 48 h and tested in a cytotoxicity assay after 24 h. A mock vector-transfected clone of each cell line was used for the control.

Cytotoxicity Assay. The lytic activity of CTLs was tested by a conventional ^{51}Cr release assay. Briefly, target cells were labeled with 100 μCi of ^{51}Cr for 1 h at 37°C, washed three times, and resuspended in AIM-V medium. Then, ^{51}Cr-labeled target cells (2000 cells/well) were incubated with various numbers of effector cells for 6 h at 37°C in 96-well microtiter plates. The radioactivity of the culture supernatant was measured by a gamma counter. The percentage cytotoxicity was calculated as follows: % cytotoxicity = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. In some cases, target cells (WiDr, MKN7, MKN28, TE4, TE8, and AZ521) were treated with 100 units/ml IFN-γ for 48–72 h before the assay. For peptide-pulsed target cells, TISI (HLA-A*2402) was incubated with 1 μg/ml peptide at room temperature for 1 h before the assay. To explore the inhibition of cytotoxicity with anti-HLA-class I mAb, TISI cells were incubated for 1 h at room temperature before the assay.

Cytokine Release. The supernatant of NY-ESO-1-specific CTLs was collected after a 24-h coculture with the various target cells and stocked at −80°C until the IFN-γ release assay. A commercially available immunoenzymetric assay kit was used (Immunotech, Marseille, France).

Inhibition of IFN-γ Productivity by Blocking mAbs. To determine the mechanism of immunological response in the recognition of antigens by the NY-ESO-1-reactive CTLs, NY-ESO-1-transfected WiDr cells were pretreated with anti-HLA class I (W6/32) or anti-HLA class II (L243; Becton Dickinson, San Jose, CA) mAbs for 1 h at room temperature. These pretreated target cells were tested for their capacity to inhibit the IFN-γ productivity by the NY-ESO-1-specific CTLs in response to the target cells. This experiment was performed in triplicate.

Statistical Evaluation. Statistical analysis was performed using the unpaired two-tailed Student's *t* test to compare

Fig. 1 Binding activity of NY-ESO-1-derived peptides to HLA-A*2402 molecule. Binding affinity of peptides to HLA-A*2402 molecule was evaluated by mean fluorescent intensity (*M.F.I.*) of cell surface HLA-A*2402 molecule on RMA-S-A*2402/K^b cells that were pulsed with peptides. HLA-A*2402-restricted, MAGE-2-derived peptide (EYLQLVFGI) was used as a positive control.

cytokine expression. Differences were considered significant when the *P* was <0.05.

Results

Binding Analysis of Synthetic Peptides to HLA-A*2402. Initially, the known sequences of NY-ESO-1 were screened for peptides containing the anchor motif for HLA-A24. Six peptides of nine residues and three peptides of 10 residues were found to contain the binding motif for HLA-A24 (Table 1). Then, the peptides were tested for binding affinity to the HLA-A*2402/K^b chimera gene. It is known that MHC class I molecules become unstable in the absence of binding peptides. Because RMA-S cells lack TAP molecules, which transport peptides into the endoplasmic reticulum, the MHC class I level on the cell surface of RMA-S cells is low compared with wild-type RMA cells. In the presence of binding peptides, MHC class I is stabilized, leading to up-regulation on the cell surface. As shown in Fig. 1, within the four peptides, NY-ESO-1-derived peptide was capable of increasing the HLA-A24 level on RMA-S A*2402/K^b cells. MAGE-2/HLA-A24 (EYLQLVFGI) peptide, which has been reported to be an HLA-A24-presentable CTL epitope, increased the HLA-A24 level as well. These data indicate that four peptides (SCLQQLSLL, LLMWITQCF, SISSCLQQL, and SGNILTIRL) have a significant binding affinity to HLA-A*2402.

Induction of Antigen-Specific CTL Responses by NY-ESO-1-Derived Peptides. We attempted to induce CTLs recognizing NY-ESO-1-derived peptide in the context of HLA-A24, which is the most frequent allele in the Japanese population. PBMCs collected from three normal HLA-A*2402-positive volunteers were tested for induction of CTLs *in vitro*. CD8$^+$ T cells sorted out from PBMCs were incubated with peptide-pulsed autologous monocyte-derived DCs, followed by additional stimulation with peptide-pulsed autologous PHA-blasts. After four stimulations, the cytotoxic activity against the peptide-pulsed target cells was examined by a ^{51}Cr release assay. Of the four peptides studied, only a high MHC binder

Table 1 HLA-A24 binding of motif containing peptides derived from the sequence of NY-ESO-1

Sequence	Position	Predictive binding score[a]
SGNILTIRL	129	7.2
SCLQQLSLL	151	6.0
FATPMEAEL	96	5.3
LLMWITQCF	158	5.0
SISCLQQL	148	4.8
TQCFLPVFL	163	4.8
RLTAADHRQL	136	8.0
RGPESRLLEF	81	7.9
LSISSCLQQL	147	7.2

[a] Predicted binding to HLA-A*2402 was determined using an algorithm that calculates the theoretical dissociation rate of the corresponding A24/peptide complexes.
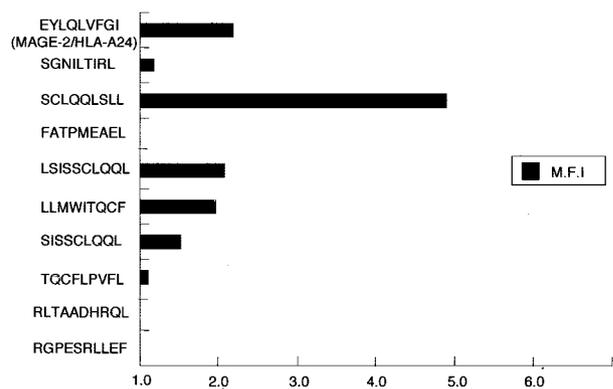
(LLMWITQCF) was able to elicit CTLs. After 28 days of culture, the induced effector cells exhibited >80% cytotoxicity against the peptide-pulsed TISI cells (HLA-A24[+]), compared with minimal cytolysis observed against TISI cells alone at the E:T ratio of 20:1 (Fig. 2*A*). In addition, the cytotoxic activity was in a peptide dose-dependent manner (Fig. 2*B*). These results indicated that the CTLs recognized the peptide in the context of HLA-A*2402 and the peptide.

Inhibition of the Recognition of CTLs by Anti-HLA-Class I Antibody. To determine whether the peptide NY-ESO-1 (158–166) (LLMWITQCF)-induced CTLs recognized

Fig. 3 Expression of the *NY-ESO-1* gene in cell lines detected by reverse transcription-PCR and agarose gel electrophoresis (*A*). PCR amplification was performed with specific oligonucleotides. Lysis of various tumor cell lines by the induced CTLs using the peptide LLM-WITQCF. The cytotoxic activity of the CTLs was assessed against SK-MEL-128 (melanoma cell line, HLA-A24[+], and NY-ESO-1^{+}), TE4 (esophageal carcinoma cell line, HLA-A2[+], and NY-ESO-1^{+}), TE8 (esophageal carcinoma cell line, HLA-A26[+], and NY-ESO-1^{−}), AZ-521 (gastric carcinoma cell line, HLA-A2[+], and NY-ESO-1^{−}), and K562, at various E:T ratios (*B*).
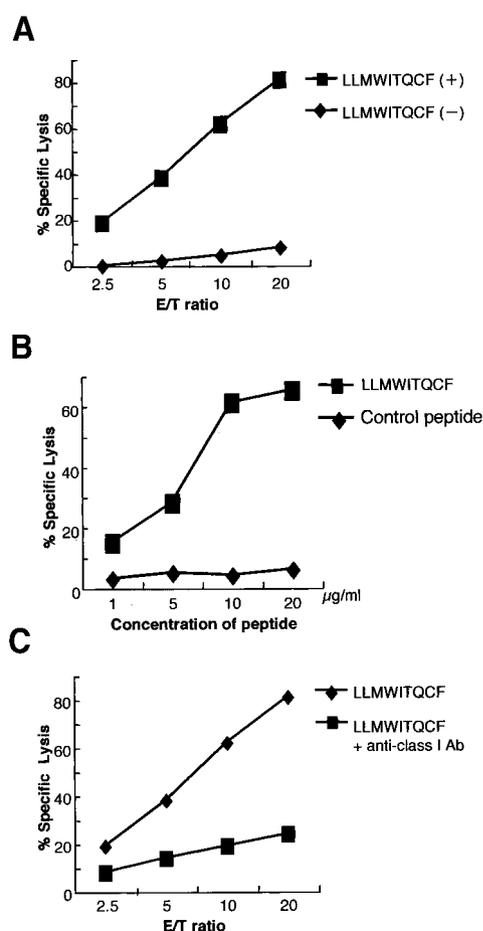

Fig. 2 The cytotoxic activity of the effector cells induced by stimulation with NY-ESO-1/HLA-A24 peptide LLMWITQCF against TISI cells pulsed with the peptide. The effector cells were obtained by stimulating CD8^{+} T cells for 28 days with mitomycin C-treated autologous PHA-blasts pulsed with the peptide. TISI cells were pulsed with or without 10 μg/ml this peptide and then labeled with [51]Cr. The cytotoxic activity against the TISI cells pulsed either with or without the peptide was assessed at various E:T ratios (*A*). The TISI cells were pulsed with different concentrations (1–20 μg/ml) of the peptide for 1 h; then the cytotoxic activity was assessed at E:T ratio = 10:1 (*B*). HLA-A*2402-restricted, MAGE-2-derived peptide (EYLQLVFGI) was used as control peptide. The recognition of induced CTLs was inhibited by the anti-HLA-class I antibody (W6/32; *C*). TISI cells were incubated with or without anti-HLA-class I antibody (W6/32) for 1 h at room temperature. The cytotoxic activity of the CTLs was determined against peptide-pulsed TISI cells at various E:T ratios by a ^{51}Cr-release assay.
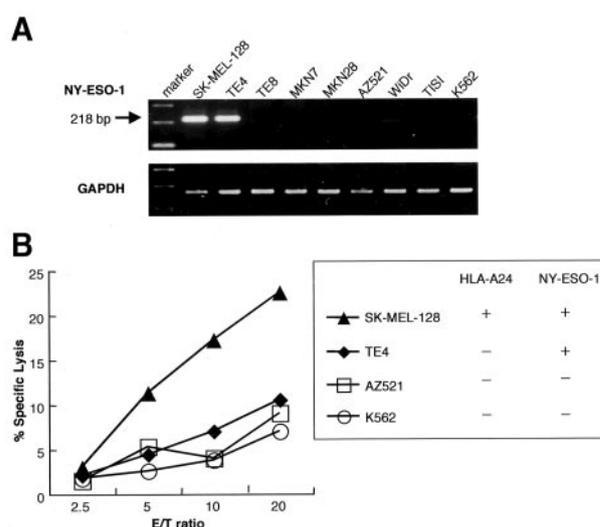
the peptide-pulsed target cells in an HLA-class I-restricted manner, the mAb against HLA-class I (W6/32) was used to block recognition by CTLs. Our results showed that the anti-HLA-class I antibody could significantly eliminate the cytotoxicity of the CTLs against peptide-pulsed TISI cells (HLA-A24[+]; Fig. 2*C*), thus indicating that the induced CTLs lysed peptide-pulsed target cells in an HLA-class I-restricted manner.

Expression of NY-ESO-1 mRNA. We analyzed the expression of NY-ESO-1 mRNA in all cell lines used in this study. The *NY-ESO-1* gene was expressed in SK-MEL-128 (the melanoma cell line, HLA-A24[+]) and TE4 (the esophageal carcinoma cell line, HLA-A2[+]). However, the expression of the *NY-ESO-1* gene was not observed in the gastric carcinoma cell line MKN7 (HLA-A24[+]), MKN28 (HLA-A31/A33[+]), AZ521 (HLA-A2[+]), the esophageal carcinoma cell line TE8 (HLA-A26[+]), the colon carcinoma cell line WiDr (HLA-A24[+]), TISI (human B-lymphoblastoid cell line, HLA-A24[+]), and K562 (Fig. 3*A*).

Antigen Specificity of the NY-ESO-1-Specific CTLs. The CTLs that were induced by peptide-pulsed, antigen-presenting cells were able to lyse SK-MEL-128 (NY-ESO-1^{+}, HLA-A24[+]). However, the cytolysis was minimal against TE4 (NY-ESO-1^{+}, HLA-A2[+]), AZ521 (NY-ESO-1^{−}, HLA-A2[+]), and K562 cells (NY-ESO-1^{−}; Fig. 3*B*). In addition, the induced CTLs had a potent cytolytic activity against HLA-A24[+] carcinoma cell lines transfected with NY-ESO-1 cDNA (WiDr/NY-ESO-1, MKN7/NY-ESO-1), whereas the CTLs were not able to lyse HLA-A24[−] or NY-ESO-1^{−} carcinoma cell lines (WiDr/Mock, MKN7/Mock, TE8/Mock, TE8/NY-ESO-1, MKN28/Mock, MKN28/NY-ESO-1; Fig. 4, *A–D*). Moreover, the in-
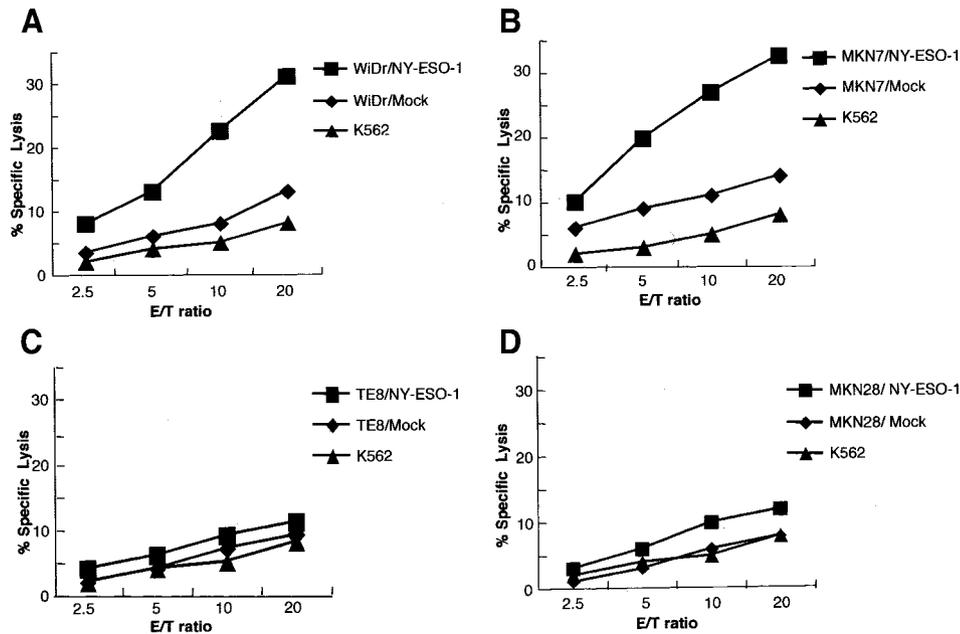
Fig. 4 The cytotoxic activity of the induced CTLs against colon carcinoma cell line WiDr (*HLA-A24*; *A*), gastric carcinoma cell line MKN7 (*HLA-A24*; *B*), esophageal carcinoma cell line TE8 (*HLA-A36*; *C*), and gastric carcinoma cell line MKN28 (*HLA-A31/A33*; *D*) transfected with plasmid pcDNA3.1 containing NY-ESO-1 cDNA, at various E:T ratios.
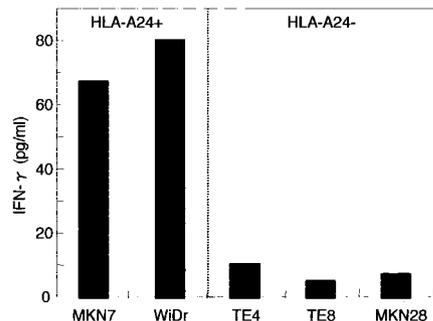

Fig. 5 NY-ESO-1 restriction of the NY-ESO-1-specific CTLs. Various NY-ESO-1⁻ human cancer cell lines that were transiently transfected with pcDNA3.1 containing NY-ESO-1 cDNA 48 h before the CTL assay were cultured with the NY-ESO-1-specific CTLs at a final E:T ratio of 10. Each supernatant was collected 24 h later, and the concentrations of IFN-γ were determined by an immunoenzymetric assay.

specific CTLs when they were cultured with NY-ESO-1-transfected WiDr cells. The levels of IFN-γ released by the NY-ESO-1-specific CTLs were significantly inhibited by anti-HLA class I mAbs but not by anti-HLA class II mAbs, indicating that the CTLs recognize the NY-ESO-1-derived epitopes in an HLA class I-restricted manner (Fig. 6).

Discussion

The therapeutic options for the treatment of patients with carcinomas are limited to three fundamental modalities: (*a*) surgical resection; (*b*) chemotherapy; and (*c*) radiation therapy. Especially against advanced carcinomas, these modalities do not yield good results. Over the past few years, the analysis of spontaneous immune responses to autologous tumors in cancer
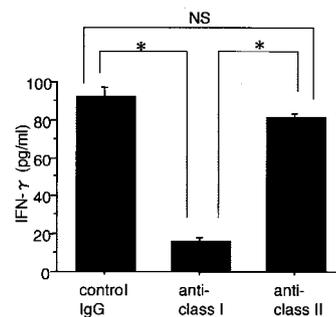
duced CTLs produced high levels of IFN-γ when cultured with two kinds of HLA-A24⁺ cancer cell lines, which were transfected with pcDNA3.1 containing NY-ESO-1 cDNA for 48 h before the experiment. On the other hand, IFN-γ production was marginal when the same CTLs were cultured with NY-ESO-1-expressing HLA-A24⁻ esophageal cancer (TE4, TE8) and MKN28 gastric cancer cell lines (Fig. 5). These results suggested that the CTLs could recognize NY-ESO-1 antigen and demonstrate potent cytotoxic activity against various types of carcinoma cells that expressed both NY-ESO-1 and HLA-A24.

HLA Class I Restriction of the NY-ESO-1-Specific CTLs. To determine the mechanism of immunological response in the recognition of antigen by the NY-ESO-1-reactive CTLs, anti-HLA class I and II mAbs were tested for their capacity to inhibit the IFN-γ productivity by the NY-ESO-1-

Fig. 6 Inhibition of specific recognition of NY-ESO-1-reactive CTLs by mAbs. NY-ESO-1-transfected WiDr cells were preincubated with anti-HLA class I or II mAbs for 1 h at room temperature. Then, the CTLs and pretreated target WiDr cells were cocultured at an E:T ratio of 10. IFN-γ concentrations in each supernatant were measured 24 h later by an immunoenzymetric assay. Data represent the mean ± SD of the IFN-γ release. ∗$P < 0.05$; *NS,* not significant.

patients has allowed the identification of several categories of tumor-associated antigens that can be the target of tumor-specific immune responses based on the recognition of tumor antigens by CTL in an MHC-class I/peptide complex-restricted manner (16, 17). Therefore, cancer-specific immunotherapy has become a very attractive fourth-modality therapeutic approach against carcinomas. Among them, one of the most relevant for the development of tumor immunotherapy is peptide-based, cancer-specific immmunotherapy using the group of the so-called CT antigens, which are expressed by tumor cells but not by most somatic adult tissues, with the exception of the testis, an immune-privileged organ (2). Therefore, the identification of T-cell epitopes from these antigens has become a critical step in the development of peptide-based immunotherapy for cancer.

A major breakthrough in the identification of T-cell epitopes was the finding that ligands of a certain MHC-molecule carry chemically related amino acids in certain positions, which led to the definition of a peptide motif for every MHC allele (18). This knowledge was rapidly used to predict potential epitopes from various antigens and became the starting point of the so-called reverse immunology, which has been the most successful strategy for the identification of T-cell epitopes (19). This approach included a four-step procedure of: (*a*) a computer-based epitope prediction from the amino acid sequence of a candidate antigen; (*b*) a peptide-binding assay to determine the affinity of the predicted peptide with MHC molecule; (*c*) the stimulation of primary T-cell response against the predicted peptides *in vitro*; and (*d*) testing of the resulting CTLs toward target cells endogenously expressing the antigen (11). With this approach, numerous T-cell epitopes have been identified from several CT antigens, including as MAGE-3 (4) and TRP-2 (20).

It has been reported that 40–50% of melanoma patients with advanced tumors expressing NY-ESO-1 possess a simultaneous antibody and CTL response against NY-ESO-1, demonstrating that NY-ESO-1 is to date the only CT antigen capable of eliciting both a humoral and cellular response in a large proportion of patients (21). The high immunogenicity of NY-ESO-1 and its broad tumor expression make this protein a very promising target for tumor-specific vaccination strategies. If NY-ESO-1 epitopes were presented by different tumor cells and recognized by CTL, then vaccines designed to boost CTL responses against NY-ESO-1 epitopes would be useful. NY-ESO-1 peptides, recognized by HLA-A*0201-restricted CTLs, have recently been described (5), and in addition, NY-ESO-1-specific CD8^{+} T-cell responses could be induced *in vivo* by immunization with these peptides (22). However, to our knowledge, there is no information on HLA-A24-restricted CTL epitopes. HLA-A2 is highly heterogeneous, and only 45% of HLA-A2[+] Japanese are of the HLA-A2.1 subtype (10). In contrast, the HLA-A24 allele is much less heterogeneous and >90% of HLA-A24 is the HLA-A*2402 subtype, which is highly expressed in Asians and in 60% of the Japanese population (10). HLA-A24 is also found in significant numbers of individuals belonging to other ethnic groups (33% in Chinese, 27% in Hispanics, 17% in Caucasians, and 9% in African-Americans; Ref. 10). In the present study, we demonstrated that an HLA-A24-binding epitope peptide derived from the amino acid sequence of NY-ESO-1 was able to elicit HLA-class I-restricted CTLs that would kill tumor cells expressing both

NY-ESO-1 and the corresponding HLA molecules. Because it will be fundamental to identify HLA-specific epitopes restricted to the most common allele for developing peptide-based immunotherapy in the general population, the identification of a novel epitope restricted by HLA-A24 represents an important addition to the peptide epitopes available for the development of a safe and effective peptide-based vaccine treatment strategy against carcinomas in Japan and worldwide.

Interestingly, our finding, the new HLA-A24 epitope, NY-ESO-1 158–166, is located very close to the known HLA-A2 epitope, NY-ESO-1 157–165 (5). It is possible that, through the action of serum proteases or APC derived proteases, extracellular antigen processing of large peptides occurs, generating MHC class I-binding epitopes for CTLs (23, 24). Other possibilities could also be considered, because there are many cleavage sites in the peptide sequence in proteasome digestion (25). It is interesting that this mechanism will be verified by *in vitro* experimental work, such as proteasome-mediated digestion analysis, in the near future.

Peptide-based cancer immunotherapy is a potentially promising new treatment modality, because it is tumor specific, less toxic, and could have a long-lasting effect. Although the results of immunotherapeutic cancer treatments have been promising in experimental models, as yet, the overall success in human trials has been modest (26), partly caused by the heterogeneous expression pattern of tumor antigens. In fact, we have reported that two kinds of CT antigens (MAGE-3 and NY-ESO-1) were heterogeneously expressed in the same tumor tissue (27). Therefore, identification of many more tumor antigens is very important to overcome the heterogeneity, expand the indication of tumor immunotherapy, and provide more powerful clinical effectiveness.

In conclusion, our results suggest that NY-ESO-1/HLA-A24 peptide, LLMWITQCF (amino acid position in NY-ESO-1 158–166), might be capable of inducing HLA-A24-restricted CD8^{+} CTL, which would be a powerful weapon in cancer-specific immunotherapy. Currently, we are now planning to perform a clinical trial of multiple peptide-based pulsed DC vaccine therapy using NY-ESO-1(158–166) (LLMWITQCF) peptide and MAGE peptides in HLA-A24[+] patients with advanced gastrointestinal carcinomas.

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Identification of HLA-A24-Restricted CTL Epitope from Cancer-Testis Antigen, NY-ESO-1, and Induction of a Specific Antitumor Immune Response

Hiroshi Yamaguchi, Fumiaki Tanaka, Mitsuhiko Ohta, et al.

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